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## More Efficient Capture of Bacteria on Nanostructured Materials

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### ABSTRACT

Nanobiotechnology is a growing area of research, primarily due to the potentially numerous applications of new synthetic nanomaterials in engineering/science. Although various definitions have been given to the word "nanomaterials" by many different experts, the commonly accepted one refers nanomaterials as those materials which possess grains, particles, fibers, or other constituent components that have one dimension specifically less than 100 nm. In biological applications, most of the research to date has focused on the interactions between mammalian cells and synthetic nanophase surfaces for the creation of better tissue engineering materials. Although mammalian cells have shown a definite positive response to nanophase materials, the evidence for bacteria interactions with nanophase materials remains for the most part a mystery. For this reason, this study determined the capture of a model bacteria (*Pseudomonas fluorescens*) on nanophase compared to conventional grain size alumina. Results provided the first evidence of increased capture of *Pseudomonas fluorescens* on alumina with nanometer compared to conventional grain sizes. Although not measured at the atomic scale, similar chemistry, crystallinity, crystal phase, and porosity was observed between nanophase and conventional alumina. For this reason, a major material property difference between nanophase and conventional alumina was reduced grain size (and perhaps associated changes in charge density) which led to increased bacteria capture and the design of better environmental filters.

### INTRODUCTION

Nanobiotechnology is a growing area of research [1], primarily due to the potentially numerous applications of new synthetic nanomaterials in engineering/science. Although various definitions have been given to the word "nanomaterials" by many different experts, the commonly accepted one refers nanomaterials as those materials which possess grains, particles, fibers, or other constituent components that have one dimension specifically less than 100 nm [2]. For example, in catalytic applications, compared to conventional grain size magnesium oxide, nanophase grain size magnesium oxide adsorbed up to ten times more organophosphorous and chlorocarbons [3, 4]. It was speculated that nanophase compared to conventional grain size magnesium oxide increased adsorption of these species due to greater numbers of atoms at the surface, a higher surface area, increased grain boundaries at the surface, and less acidic OH- groups (due to a much larger proportion of edge sites for the nanophase magnesium oxide to cause delocalization of electrons) [3, 4]. Such novel surface properties contribute to the noted increased wettability of nanophase compared to conventional ceramics [5] and, consequently, have lead to the investigation of nanophase materials in biological (or aqueous) environments.

In biological applications, most of the research to date has focused on the interactions between mammalian cells and synthetic nanophase surfaces for the creation of better tissue engineering materials [6-8]. Although mammalian cells have shown a definite positive response to nanophase materials, the evidence for bacteria interactions with nanophase materials remains at large a mystery. This is despite theory which strongly suggests the use of nanophase materials as more effective filters to remove bacteria from contaminated media such as water and soil.

## **EXPERIMENTAL DETAILS**

### **Materials**

As a model nanophase material, alumina was used in the present study. Nanophase alumina (23 nm size) particles were obtained from Nanophase Technologies, Corp. (Romeoville, IL). Nanophase ceramic particles were used as supplied by the manufacturer. Nanophase alumina particles were first loaded into a steel-tool die to obtain compacts for use in bacteria experiments. A serial pressure cycle (from 0 - 10 GPa over 11 minutes) using a simple uniaxial, single-ended hydraulic press (Carver, Inc.) was utilized to press all alumina particles into compacts. Particles were pressed in air at room temperature. After pressing, nanophase alumina was heated (in air at 10°C/min.) to 1000°C and sintered at this temperature for 2 hours. Sintering at this temperature created crystalline alumina with nanometer grain sizes. To obtain larger conventional grain size (i.e., control) compacts, alumina nanophase compacts were heated (in air at 10°C/min.) to 1200°C and sintered at this temperature for 2 hours. Compacts were prepared with an 11 mm diameter, and were fixed to the center of a glass slide (Fisher Scientific) with silicon-based sealant. Compacts were rinsed with sterile deionized, distilled water and were then autoclaved before use in cell culture experiments.

### **Material Characterization**

Nanophase and conventional alumina compacts were analyzed for surface area and surface roughness using a NanoScope IIIa Atomic Force Microscope (AFM; Digital Instruments Inc.). Specifically, height images of each sample were captured in the ambient air at 15-20% humidity at a tapping frequency of approximately 300 kHz, and a frequency near the resonance of the cantilever. The scan field of view was 5  $\mu$ m x 5  $\mu$ m using a scanning rate of 1 Hz and 256 scanning lines. The resulting height images were analyzed using NanoScope imaging software (Digital Instruments, Inc.).

### **Bacteria Interactions**

To determine bacteria response to nanophase compared to conventional alumina, a pure culture of *Pseudomonas fluorescens* MF0 was donated by Professor Sylvie Chevalier (Université de Rouen, Evreux, France) and was used as a model bacteria cell line in these experiments. *P. fluorescens* is ubiquitously distributed in water and soil, and is frequently isolated from environmental and food specimens. Pure cultures of *P. fluorescens* MF0 were grown at room temperature (22  $\pm$  0.5 °C) with continuous shaking at 150 rpm in trypticase soy broth (Difco). Cultures were harvested when the optical density at 600 nm was approximately 1.00, which was determined to be the mid-log phase for this strain. The optical density was determined using a

Perkin Elmer Lambda 20 Spectrometer (Perkin Elmer Inc., USA). The cultures were harvested by four consecutive stages of centrifugation (6000 g for 10 min and at 4 °C) and re-suspended in phosphate buffer saline (PBS) (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, and 2 mM KH<sub>2</sub>PO<sub>4</sub>). The centrifugation step is necessary to remove the growth substrate from the solution so that there was no significant bacterial growth during the adhesion assessment study. The final suspensions from centrifugation vials were combined to a volume of 300 ml with cell concentrations equal to approximately 5×10<sup>8</sup> cells/mL. The PBS-buffered suspensions were allowed to equilibrate to room temperature before adhesion testing.

For each bacteria capture batch assay, alumina compacts of the two topographies (either nanophase or conventional) were tested in parallel. Four treatments were assessed (nanospherical alumina without fibronectin, nanospherical alumina with fibronectin, conventional alumina without fibronectin, and conventional alumina with fibronectin), along with appropriate controls. Fibronectin is an adhesive protein that mediates *P. fluorescens* capture [9]. Alumina compacts were coated with 5 µg/ml of fibronectin (Sigma) in PBS for 8 hours overnight at room temperature. The capture experiments were carried out in triplicate in 50 mL centrifuge vials. First, 50 mL of the centrifuged bacterial suspension in PBS (or filtered, sterile water for the control) was placed into each vial. Then, a glass slide containing either nanophase or conventional alumina was gently inserted into the vial. Caps were secured tightly on the vials to prevent leakage. The vials were immediately placed on a slantwise rotator and slowly rotated at 12 rpm. After 1 hour, the vials were opened and the adhesion of bacteria onto the surface was assessed by epi-fluorescence microscopy. After removal of the slide, it was placed in a PBS-buffered fixative for 15 minutes. The slides and compacts were then rinsed gently with PBS and air-dried. Exactly 100 µL of DAPI (4'-6-diamidino-2-phenylindole) (10 mg/L) was applied to each compact and then the slide was incubated in the dark for 20 minutes. Excess DAPI was rinsed from the slide, the compacts were allowed to air-dry and covered with a drop of non-fluorescence immersion oil (type FF, Cargille Laboratories, Inc.) followed by a glass coverslip.

Bacterial cell counts were conducted with a Nikon E 800 Bio-Research Microscope (Nikon Instrument, Inc. USA) at 1000x magnification. The FITC filter cube was used to select the excitation wavelength range for optimal excitation. Approximately 15 different fields of 0.01 mm<sup>2</sup> for each sample were randomly selected to allow for statistical significance. Bacteria capture was normalized to alumina compact surface area. Experiments were run in triplicate at repeated at least three separate times. All data was analyzed by standard t-tests with statistically differences between means determined at  $p < 0.05$ .

## RESULTS

### Material Characterization

Although not measured at the atomic level, results of this study provided evidence that the nanophase and conventional (that is, particle sizes larger than 100 nm) alumina compacts possessed similar chemistry, crystalline phase, no-porosity, and altered primarily in surface feature size or degree of nanometer surface roughness. The nanophase alumina compacts possessed significantly higher surface roughness values and had approximately 30% more surface area when compared to conventional alumina (Figure 1).

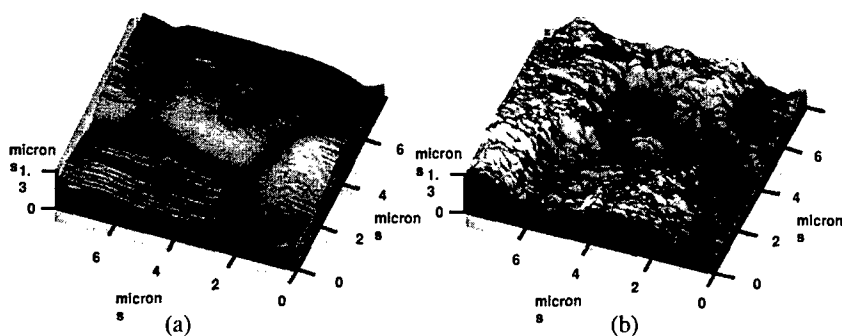


Figure 1. Atomic force micrographs of (a) conventional and (b) nanophase alumina surfaces.

### Increased Bacteria Cell Capture on Nanophase Alumina

Most importantly, the results of this study provided the first evidence of increased bacteria cell capture on alumina that alters only in degree of nanometer surface roughness. For this reason, a size-dependent biological property for alumina was elucidated for the first time with bacteria in this study. Specifically, the nanophase alumina surface captured significantly ( $p < 0.01$ ) higher *P. fluorescens* than the conventional surface either in the presence or absence of adsorbed fibronectin (Figure 2). In fact, 2 and 1.4 times the amount of *P. fluorescens* were captured on nanophase compared to conventional alumina with and without pre-adsorbed fibronectin, respectively. Decreased bacteria remaining in the supernatant was also observed for nanophase alumina, thus, confirming the present data of increased capture on nanophase alumina. Clearly, this is much greater than the 30% increase in surface area of nanophase compared to conventional alumina and, thus, indicates that properties other than increased surface area (for example, greater surface reactivity as discussed later) are enhancing the capture of *P. fluorescens*.

The surface with the highest level of adhesion was the nanophase alumina with pre-adsorbed fibronectin. There was no significant difference between the conventional surface with pre-adsorbed fibronectin and the nanophase surface without pre-adsorbed fibronectin. The conventional surface without fibronectin had the lowest level of bacterial adhesion. Although it was expected that within each group, *P. fluorescens* capture would be greater when fibronectin was pre-adsorbed (since fibronectin enhances adhesion of bacteria [9]), it was surprising to find that similar bacteria capture was measured on conventional alumina pre-adsorbed with fibronectin compared to nanophase alumina not pre-adsorbed with fibronectin. This, undoubtedly, speaks to the unprecedented influence nanophase alumina has on bacteria capture.

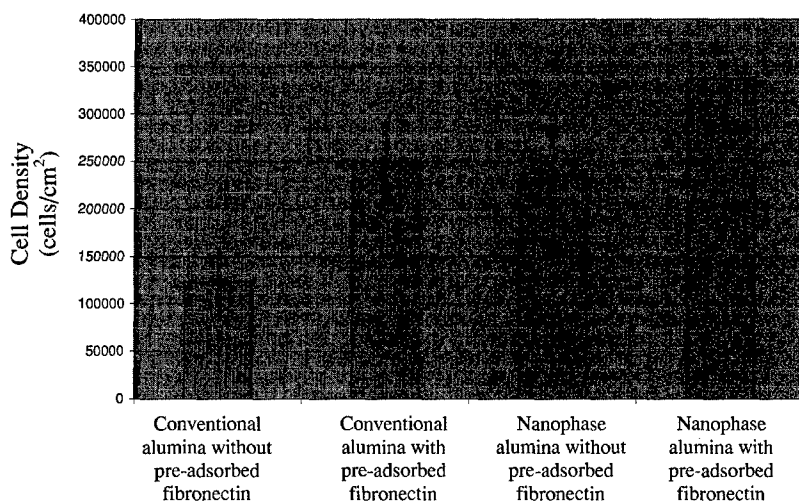


Figure 2. Increased *P. fluorescens* capture on nanophase compared to conventional alumina with and without pre-adsorbed fibronectin. Bacteria capture was normalized to surface area. Data = mean  $\pm$  SEM;  $n = 3$ ; \*  $p < 0.01$  compared to respective alumina with and without pre-adsorbed fibronectin.

## DISCUSSION

As previously mentioned, nanophase ceramics, metals, polymers, and composites all increase in vitro functions of mammalian cells important for the efficient regeneration of bone, cartilage, vascular, and bladder tissue [5-8]. Some of these differences have been extreme. For example, 4 times the amount of in vitro bone synthesis was observed on nanophase compared to conventional alumina. These are the same two substrates studies here. In fact, greater osteoblast adhesion was observed specifically on alumina samples with grain sizes less than 60nm [6]. Future studies will be needed to determine at which select alumina grain sizes bacteria capture is enhanced as pertaining to the results of this study. In addition, at this time it is unclear what underlying surface properties (topography versus changes in surface charge density) are enhancing bacteria capture. Future studies will be needed to further investigate this.

Recent literature reports have implicated initial protein interactions with nanophase materials as the underlying parameter that results in increased mammalian cell function [5, 11]. That is, compared to larger grain-size, conventionally-structured alumina, greater adsorption of biological serum on nanospherical alumina was demonstrated after four hours. In fact, when normalized to surface area, 55% more serum (which contains numerous proteins such as laminin, collagen, fibronectin, and vitronectin) adsorbed on alumina surfaces when grain sizes were reduced into the nanometer regime [5]. It is important to note that protein adsorption did not linearly correlate with increases in surface area [5]. In fact, select protein adsorption was observed at grain boundaries. This may help to explain why nanophase alumina with fibronectin

only enhanced bacteria capture 1.4 times while conventional alumina with fibronectin enhanced bacteria capture 2 times compared to respective substrates without fibronectin.

In addition, other literature reports indicated that the conformation or bioactivity of these proteins once adsorbed are also enhanced on nanophase compared to conventional alumina surfaces [7]. Specifically, mammalian cell-adhesive epitopes in fibronectin (for example, Arginine-Glycine-Aspartic acid or RGD) were exposed to a greater extent when adsorbed on nanophase compared to conventional alumina surfaces [7]. Increased adsorption and bioactivity of these proteins may be related to the greater surface reactivity (due to the greater number of atoms, increased electron delocalization, higher numbers of grain boundaries at the surface, percentage of defects on the surface, etc.) inherent for nanophase compared to conventional materials. Such studies were the first to provide a mechanism for the documented size-dependent relationship of increased in vitro bone tissue regeneration on ceramics with grain sizes decreasing into the nanometer regime.

## CONCLUSIONS

In summary, although there has been speculation about bacterial interactions with nanophase materials, very little research has been reported in this area. In this manner, this study represents one of the first (if not the first) to demonstrate the benefits of using nanophase materials in environmental applications for the efficient capture of bacteria from waste water, soil, etc. This study provides evidence towards an important size-dependent (or associated charge density change) biological property relationship for enhancing bacteria capture. Thus, this study demonstrated another novel property of nanophase ceramics: increased capture of bacteria.

## ACKNOWLEDGEMENTS

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